1978

Specificity of Antisera Against Hordeum Vulgare Ribonuclease and Serological Quantitation of the Enzyme in Tissue Extracts

Steven Bryan Hawthorne

SPECIFICITY OF ANTISERA AGAINST *HORDEUM VULGARE* RIBONUCLEASE AND SEROLOGICAL QUANTITATION OF THE ENZYME IN TISSUE EXTRACTS

BY

STEVEN BRYAN HAWTHORNE

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Chemistry, South Dakota
State University
1978

SPECIFICITY OF ANTISERA AGAINST *HORDEUM VULGARE* RIBONUCLEASE AND SEROLOGICAL QUANTITATION OF THE ENZYME IN TISSUE EXTRACTS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

D. G. Kenefick Date
Thesis Adviser

H. G. Hecht Date
Head, Chemistry Dept.

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to Dr. Don Kenefick, my major professor and adviser for his guidance and support during this period of study. Dr. Robyn Hillam's assistance in learning, developing, and troubleshooting the immunological techniques involved in this study is greatly appreciated as well as her time spent in reviewing the manuscript. The excellent laboratory assistance from Mrs. Teresa DeBoise is sincerely appreciated. I also wish to thank the Department of Chemistry for assistantship support.

SPECIFICITY OF ANTISERA AGAINST *HORDEUM VULGARE* RIBONUCLEASE AND SEROLOGICAL QUANTITATION OF THE ENZYME IN TISSUE EXTRACTS

S. B. Hawthorne[1], R. P. Hillam and D. G. Kenefick[2]

ABSTRACT

Specific rabbit antisera against purified seedling RNase I from both a hardy (Dicktoo) and less-hardy (Tennessee Winter) cultivar of winter barley was obtained using a 1 mg injection schedule. Both antisera formed a single precipitin band on double immunodiffusion and immunoelectrophoresis when reacted with the homologous crude tissue extract.

RNase antigen from either cultivar was highly cross-reactive with both antisera. Passive hemagglutination inhibition was used in an attempt to distinguish purified RNase from both cultivars. A consistent difference in anti-RNase serum specificity between cultivars was shown, but the difference observed by this method was not sufficient to conclude structural differences between the two RNase antigens. Immunodiffusion and rocket immunoelectrophoresis were used to qualitatively and quantitatively test the cross-reactivity of protein preparations from various members of the species *Hordeum* as well as unrelated species of grasses. Both antisera preparations were sufficiently specific for barley RNase that soluble protein preparations from species other than *Hordeum* showed no cross-reactivity with either antiserum. Only certain species of *Hordeum* were shown to be cross-reactive.

A quantitative method for the determination of RNase in unpurified tissue extracts was developed using a modification of rocket immunoelectrophoresis. The technique modifications include a template-

reservoir which allowed detection of 250 ng of RNase in sample volumes up to 50 ul. The quantity of RNase in unpurified protein extracts from a hardy and a less-hardy cultivar of barley was shown to be the same even though the RNase activity has been shown to differ greatly between the two cultivars.

[1]Contribution of the South Dakota Experiment Station, Brookings, SD, 57007, as Journal Article No. ____. Part of a thesis submitted by the senior author in partial fulfillment of requirements for the M.S. degree in Chemistry.

[2]Collaborative research from the Department of Chemistry, Microbiology and Plant Science, respectively. Date received

INTRODUCTION

Variation in activity of RNA-degrading enzymes among cultivars of winter barley has been reported (4). Wilson has described the biochemical properties of a number of plant nucleases. He pointed out the need for more definative information on these enzymes as an aid in describing specific roles in RNA degradation (15). In pursuit of this objective, Wong (16) purified a RNase from barley seedlings. The purified enzyme from each of two barley cultivars showed different specificities when evaluated with dinucleoside assays. These results support data showing different enzymatic activities of electrophoretically separated RNase in tissue extracts of the two cultivars (4).

Pitt (8) found serology to be a useful tool in evaluating contaminant proteins in RNase preparations from potato. He also used radial immunodiffusion to quantitate potato RNase in unpurified tissue extracts.

The purpose of this research was to evaluate the purification method described by Wong (16) for protein contaminants in the RNase fraction using serological techniques. Additionally, it was desirable to learn about the specificity of the antisera prepared against H. vulgare RNase toward the enzyme from other cereals, including other Hordeum species and cultivars within H. vulgare. Finally, rocket immunoelectrophoresis was explored as an alternative to radial immunodiffusion as a method to evaluate the quantity of RNase in tissue extracts.

MATERIALS AND METHODS

Protein Sample Preparation: Ribonuclease was purified from both a hardy (Dicktoo) and a less-hardy (Tennessee Winter) cultivar of winter barley (Horduem vulgare L.) by the method of Wong (16). Seedlings were germinated 4 days in the dark at 25 C. Two hundred grams of shoot tissue was excised from the seed, wrapped in aluminum foil, and immediately frozen in dry ice. The frozen tissue was ground in a mortar, suspended in extraction buffer, and mixed with 100 g of insoluble polyvinylpyrolidone. The suspension was filtered and the resulting filtrate was centrifuged at 105,000g for 150 minutes. The pooled supernatant was desalted on Sephadex G-25 and the protein fraction was subjected to Sephadex DEAE-A 50 chromatography. The anionic protein fraction (hereafter referred to as the APF) was next purified by preparative electrophoresis (16), again concentrated by anion exchange chromatography, and finally separated by molecular exclusion chromatography (Bio-Gel P-100). Samples were desalted after preparative centrifugation and each anion exchange step. RNase fractions were identified by activity determinations (10). Para-nitrophenylphosphate was used to detect acid phosphomonoesterase activity (1). Approximately 250 ug of purified RNase was obtained from 200 gm of 4-day-old shoots. Purified RNase from Dicktoo and Tennessee Winter will hereafter be designated $RNase_D$ and $RNase_{TW}$, respectively.

Small scale soluble protein samples were prepared by excising 3 g of four-day-old seedlings, freezing immediately in dry ice, suspending in 9 ml of extraction buffer, and centrifuging at 144,000g for 150

minutes. Five ml of the soluble protein was desalted on Sephadex G-25 and six ml of the eluant showing the highest absorbance at 280 nm was saved. All soluble protein samples were frozen until used. Protein was determined by the Lowry method (7).

RNase activity was determined according to Tuve & Anfinsen (10) as modified by Wong (16). The assay solution contained 0.05 M Na cacodylate (pH 5.5, adjusted with acetic acid), 0.16 M KCl, 0.2 ml of the RNase sample, and 1 mg of yeast RNA (Sigma, type X1). Total assay volume was 2.5 ml. Each sample was incubated for 30 minutes at 37 C. The reaction was stopped with 0.5 ml of 17% perchloric acid containing 0.05% uranyl acetate. The acid soluble portion was diluted 1:20 and measured at 260 nm.

The degradation of para-nitrophenylphosphate (Sigma 104) was measured according to Anraku (1) with following modifications: Assay solution contained 0.05 M acetate buffer (pH 5.0), 5 mM Sigma 104, and 0.2 ml of the enzyme sample. Total assay volume was one ml. Each sample was incubated for 30 min. at 37 C before the reaction was stopped by the addition of 0.4 ml of 0.4 N NaOH. Samples were read undiluted at 420 nm. Para-nitrophenylphosphate degrading activity shall hereafter be referred to as acid phosphomonoesterase activity.

Antibody Production: Antisera against RNase isolated from each cultivar was prepared as follows; 1 mg purified RNase protein (9) in 1.5 ml of 0.05 M tris, pH 7.8, was emulsified with 1.5 ml of Freund's complete adjuvant. This emulsion was equally divided and injected into five sites of 2-3 kg New Zealand white rabbits (3 subcutaneously,

in the axillary regions and nape of the neck; and 2 intramuscularly, in the hind flanks). One month after injection the rabbits were trial bled from the marginal ear vein. After useable antibody levels were reached, approximately 40 ml of blood was drawn per week from each rabbit by cardiac puncture following an 18-hour fasting period. An intravenous booster injection using 85 ug of the homologous antigen was administered after the antiserum titer had fallen below 500. Antisera against $RNase_D$ and $RNase_{TW}$ shall hereafter be designated anti-$RNase_D$ and anti-$RNase_{TW}$, respectively.

Titer Determination: Antibody titer was determined by the passive hemagglutination method described by Kwapinski (5). A suspension of 1.25% formalized sheep red blood cells (srbc) were sensitized with the homologous RNase antigen. Formalized srbc, washed and suspended to 2.5% in phosphate buffered saline (PBS) pH 7.2, were mixed with equal volume of tannic acid (diluted 1:2000 in PBS pH 7.2) and incubated for 10 minutes at 37 C. The cells were then washed in PBS pH 7.2 and resuspended to 2.5% concentration in PBS pH 6.4. An equal volume of 30 ug/ml solution of the homologous RNase antigen in PBS pH 6.4 was added and the resulting 1.25% srbc solution was stored at 4 C until used.

All antisera and normal rabbit serum (NRS) used for passive hemagglutination and passive hemagglutination inhibition were inactivated at 56 C for 30 minutes. Subsequently, each ml of antisera and NRS was absorbed with 0.1 ml packed formalized srbc for 20 minutes at room temperature. Serial two-fold dilutions of each antiserum were

made in 0.025 ml of 1:100 NRS in microtiter plates. Sensitized srbc (1.25%) were washed and resuspended to 1.25% in 1:100 NRS. The washed and resuspended srbc were then added to each well on the microtiter plate. Plates were allowed to incubate overnight at room temperature and routinely read after approximately 18 hours. The titer is reported as the reciprocal of the highest antiserum dilution which yielded any agglutination of the sensitized srbc.

Passive Hemagglutination Inhibition: The microtiter method of passive hemagglutination inhibition described by Kwapinski (5) was used to determine the specificity of the two antisera for both the homologous and heterologous RNase antigen. Each antiserum was tested by the following procedure: Diluted antiserum (0.025 ml) was added to a serial two-fold dilution of purified RNase in 1:50 NRS (0.025 ml) such that the final antiserum concentration was equal to the maximum antiserum dilution that would yield complete agglutination of the sensitized srbc. The microtiter plates were then incubated for 2 hours at 37 C. After incubation 0.025 ml of 1.25% sensitized srbc in 1:50 NRS was added to each well. Plates were briefly shaken and read the next day. The lowest concentration of antigen which resulted in any inhibition of complete agglutination of the sensitized srbc was determined.

Double Immunodiffusion: A modification of the micro template method described by Crowle (3) was used. Microscope slides (25 x 75 mm clay Adams "Gold Seal") were precoated with 0.1% agarose and 0.05%

glycerine in double-glass distilled water. Spacers for the gel casting were made by placing 2 layers (approx. 0.5 mm) of Scotch #88 electrical tape on each end of the slide.

Agarose was dissolved to 0.75% in tris-barbitol, pH 8.6, 1/2 strength Gelman high resolution buffer, and cast as a uniform layer on the slide with the aid of a top casting slide rinsed with 1:200 Kodak photoflow. After the agarose had solidified the top slide was removed, the template (1" x 1" x 3/16") was slid into position, and sample wells filled (approx. 75 ul of antiserum and 50 ul antigen). Samples were allowed to diffuse for 24 to 72 hours at 4 C in a water-saturated atmosphere. Unreacted protein was removed by soaking the slide overnight in physiological saline. Slides were stained with 0.2% napthol blue black in 5:5:1 methanol:water:acetic acid (HAc) for ten minutes and exhaustively destained in 4% HAc.

Immunoelectrophoresis: Microscope slides (25 x 75mm) were prepared as above except 3 layers of electrical tape (approx. 0.75 mm) were placed lengthwise along both edges of the slide. Agarose was dissolved to a 0.75% concentration in a 1:4 dilution of 0.100 M phosphate buffer (Na_2PO_4-KH_2PO_4, pH 7.6) and cast as previously described. The undiluted buffer was used for electrophoresis. Samples (approx. 20 ul) were applied to sample wells (3 mm diameter holes punched in the gel) located 30 mm from the cathode end of the slide. Samples were electrophoresed at 100 V until the marker dye (bromophenol blue) had migrated from the sample well to the anode end of the slide. Agarose from a center trough (3 x 65 mm) was removed, the trough was

filled with antisera, and the antisera was allowed to diffuse into the gel in a water-saturated atmosphere for 24 hours at 4 C. Slides were soaked in physiological saline for one week to remove unreacted protein and stained as discussed above.

Rocket Immunoelectrophoresis: Rocket immunoelectrophoresis (2,6,11) was used to quantitate RNase with the following modifications. The method used for immunoelectrophoresis slide preparation was followed except 50 x 75 mm microscope slides were used. Equal volumes of diluted antisera (dilution will vary with antisera activity) and 2% agarose (both in 1:4 electrophoresis buffer) were mixed after both solutions were brought to 56 C. The resulting gel solution was pipetted onto the warmed, taped slide and a warmed plexiglass template-reservoir was lowered carefully onto the tape strips. The 2" x 2 3/4" x 3/16" plexiglass template-reservoir was used to increase the sample volume from 0.4 ul (the maximum sample volume possible when using only the wells punched in the thin gel) to 50 ul. The 6 funnel-shaped wells drilled in the template-reservoir were placed at the cathode end of the slide. After the gel solidified, all agarose was aspirated from the 6 funnel-shaped wells using a Pasteur pipette thus forming a 1.5 mm hole at the bottom of the sample well. Samples (1 to 50 ul) were applied in the wells using a 50 ul Hamilton syringe. Electrical contact was made on each end of the gel slab with filter paper which dipped into the buffer chambers. A 40 V potential was applied during sample application to minimize diffusion spreading. Sample runs were at 100 V for five hours at 4 C. After electrophoresis the template-

reservoir was removed and the gel-covered plates were soaked overnight in physiological saline and stained as previously described.

RESULTS & DISCUSSION

RNase Purification and Identification: Pitt reported difficulty in separating potato RNase from acid phosphomonoesterase (8). A purification schedule which ended with P-100 gel filtration allowed us to prepare barley RNase that was free of acid phosphomonoesterase activity. The yield for both cultivars was approximately 250 ug RNase from 200 g of shoot tissue. The identification of the purified RNase was based upon Wilson's criteria for dividing plant nucleases into three catagories; nuclease I, RNase I and RNase II (12,13,14). $RNase_{TW}$ and $RNase_{D}$ were not inhibited by EDTA as has been reported for nuclease I (16). Both purified RNase enzymes corresponded in electrophoretic mobility to RNase I from corn roots (16,12,13). Molecular weight, determined by Bio-Rad P-30 indicated the molecular weight of both $RNase_{TW}$ and $RNase_{D}$ to be approximately 22,000 (unpublished data) which falls within the 20,000-25,000 range reported for RNase I (14). Wong (16) reported the pH optimum for $RNase_{TW}$ and $RNase_{D}$ to be pH 5.4. Wilson (14) reports a range of pH 5.0 to 6.0 for RNase I, pH 6.7 for RNase II. On the basis of the data listed above we have tentatively determined $RNase_{TW}$ and $RNase_{D}$ be RNase I.

Antibody Production: The 1 mg RNase injection scheme was successful in producing sufficient titers against RNase from both cultivars. The rabbit injected with $RNase_{TW}$ had a passive hemagglutination titer of 500 after one month. The titer rose to a high of 4,000 six weeks after the initial injection and fell below 500 at eight weeks. At this time the 85 ug RNase booster was given but no

significant increase in titer was realized. The rabbit injected with Dicktoo RNase had a titer of 4,000 at four weeks. This titer fell slowly to below 500 at thirteen weeks. The 85 ug RNase booster was then given and yielded a slight increase in titer.

Pitt reported using 5.75 mg of potato RNase per rabbit to obtain antibody (8). Although Pitt reported no passive hemagglutination titer values that would permit a comparison of antisera, it appears that a 1 mg RNase injection scheme is adequate when limited quantities of antigen are available. The titer we obtained was sufficient for the serological tests conducted.

Specificity of Antisera: Double immunodiffusion and immuno-electrophoresis were used to determine the specificity of both antisera for RNase. Both antisera yielded single precipitin bands for both the purified homologous RNase and the homologous APF sample. RNase and APF samples showed immunological identity for both Dicktoo and Tennessee Winter antigen-antibody systems. Both anti-$RNase_D$ and anti-$RNase_{TW}$ failed to develop a precipitin band against RNase-free acid phosphomonoesterase from the Tennessee Winter cultivar indicating the purified RNase samples used for injection were free of acid phosphomonoesterase. The difficulty of this enzyme contaminating RNase, as reported by Pitt (8), was not encountered in this study. Tennessee Winter acid phosphomonoesterase was chosen for this test due to more complete separation from RNase during purification (16).

Immunoelecetrophoresis permitted characterization of antigen by its elecrophoretic mobility. Both anti-$RNase_D$ and anti-$RNase_{TW}$ gave

single precipitin bands against the purified homologous RNase and the homologous APF sample. In both the Tennessee Winter and Dicktoo antigen-antibody systems the antigenic species in the APF sample corresponded in electrophoretic mobility and precipitin band shape to the purified RNase antigen. The precipitin bands from APF_D and APF_{TW} are shown in Figure 1.

The data obtained from both immunodiffusion and immunoelectrophoresis indicate both anti-$RNase_{TW}$ and anti-$RNase_D$ to be monospecific for RNase I. If a significant quantity of non-RNase protein were present in the purified RNase sample used for injection, one would expect antibody to be formed against such species. Only one antigen-antibody system was detected with both immunodiffusion and immunoelectrophoresis indicating the RNase was free of any significant quantities of non-RNase protein. This data therefore indicates that the purification scheme reported by Wong (16) successfully eliminated non-RNase protein.

To check the species specificity of anti-$RNase_{TW}$ and anti-$RNase_D$, both antisera were tested by double immunodiffusion against soluble protein samples of spring barley (Primus II), winter wheat (Winoka), spring wheat (ERA), winter rye (Von Lockow) and spring oats (Wright). Only the Primus II antigen formed a precipitin band with both antisera. The lack of cross-reactivity shown by double immunodiffusion might be due to the low concentration of RNase in the winter and spring wheat, rye, and oat samples rather than to antigenic dissimilarity. The potential for greater sensitivity using rocket immunoelectrophoresis

encouraged us to test this possibility. Soluble protein samples (50 ul) of spring barley, winter and spring wheat, rye and oats were electrophoresed against anti-$RNase_{TW}$ and against anti-$RNase_D$. Again, characteristic precipitin rockets were obtained only from spring barley, indicating that anti-$RNase_{TW}$ and anti-$RNase_D$ are specific for H. vulgare RNase (Table 1).

Soluble protein samples derived from several species of Hordeum were also tested for reactivity against anti-$RNase_{TW}$ and anti-$RNase_D$ by rocket immunoelectrophoresis. All antigen samples were 50 ul in volume. Four of the Hordeum species showed typical precipitin rockets (Table 2). Three species showed markedly lower rocket heights than the Tennessee Winter and Dicktoo samples, and two species showed no reactivity at all. Results were the same for either source of antisera.

Cross-reactivity of Dicktoo and Tennessee Winter Antigens: Purified $RNase_D$, purified $RNase_{TW}$, APF_D and APF_{TW} samples were all found to be cross-reactive with the non-homologous anti-RNase serum. Precipitin bands showed serological identity for all 4 RNase samples against both anti-$RNase_D$ and anti-$RNase_{TW}$ (Figure 1). Serological identity shows that the species possess similar antigenic determinant sites, but does not conclusively indicate that the species are physiochemically identical (3). A more sensitive determination of whether $RNase_D$ and $RNase_{TW}$ are serologically identical species is therefore needed.

Passive Hemagglutination Inhibition: Wong (16) has shown

differences in dinucleoside specificity between $RNase_D$ and $RNase_{TW}$. The two enzymes were indistinquishable by double immunodiffusion and immunoelectrophoresis. A more sensitive technique, passive hemagglutination inhibition, was chosen to investigate possible serological differences between the two enzymes. Double immunodiffusion required approximately 50 ul of a 100 ug/ml purified RNase sample for precipitin band formation. When using anti-$RNase_{TW}$ the minimum inhibiting concentration of $RNase_D$ was 2.0 ug/ml while for $RNase_{TW}$ it was 0.50 ug/ml indicating a very slight preference of the anti-$RNase_{TW}$ for $RNase_{TW}$. Anti-$RNase_D$ could not differentiate between the purified RNase from the cultivars. Even though a reproducible relative difference was detected between RNase from the two cultivars, it was concluded that this method was not adequate to prove that a structural difference exists for the RNase from the two cultivars.

Rocket Immunoelectrophoresis: Results from RNase activity measurements in tissue extracts are complicated by the presence of non-specific nucleases. A quantitation method specific for RNase in unpurified samples would help in understanding the role of RNase in RNA degradation. Pitt (8) has explored the use of radial immunodiffusion to quantitate potato RNase. However, poor reproducibility and the fact that any error in measuring precipitin rings is squared in data interpretation suggested a need to explore another technique as an analytical method. Rocket immunoelectrophoresis initially appeared to be a more suitable technique for quantitating RNase content of tissue, but the comparatively high antigen concentrations needed and

the small sample volumes previously permissible prohibited the direct use of this technique (2,6,11). Since the sample is electrophoresed exhaustively it was speculated that the addition of a sample reservoir which would accommodate larger volumes might make this method suitable for dilute samples. The use of a template-reservoir allowed the use of sample volumes of at least 50 ul.

Kenefick et al. (4) has shown the enzymatic acitivity of RNase I in soluble protein samples from 4-day-old tissue to be much lower in hardy cultivars (e.g. Dictoo) than less-hardy cultivars (e.g. Tennessee Winter). These results could indicate either lower quantities or lower specific activity of RNase I in the hardy cultivar. Rocket immunoelectrophoresis data was plotted to develop a standard curve relating log ng RNase vs. rocket height for purified samples of $RNase_D$ and $RNase_{TW}$ using the homologous antiserum (Figure 2). Linear results of the plotted data were obtained for both cultivars. Using this method, the quantity of RNase I in each cultivar was shown to be essentially equivalent, indicating that the specific activity of RNase I from 4-day-old tissue is much higher for Tennessee Winter than for Dicktoo (Table 3). Therefore, genetic control of RNase in the two cultivars appears to be manifested by activity rather than production of the enzyme.

SUMMARY

Our work has shown that it is possible to produce a monospecific antisera to barley RNase with less purified enzyme than reported by Pitt (8). Single precipitin arcs were produced on both immunodiffusion and immunoelectrophoresis plates when soluble protein samples from Tennessee Winter and Dicktoo were tested against either antisera. The monospecificity indicates that the purification schedule reported by Wong (16) appears adequate for eliminating protein contaminants from RNase.

The lack of cross-reactivity between either antisera and soluble protein samples from non-Hordeum species and two of the Hordeum species tested indicates that the RNase in these tissues was serologically different from $RNase_{TW}$ and $RNase_{D}$. Possible structural or conformational differences between $RNase_{TW}$ and $RNase_{D}$ were suggested by passive hemagglutination inhibition. However, the differences shown by this method were too slight for any conclusions to be drawn without further investigation.

The successful adaptation of rocket immunoelectrophoresis to include a template-reservoir allowed us to specifically quantitate RNase in unpurified samples in concentrations as low as 0.25 ug/50 ul (5 ppm). This technique allowed us to show that RNase I activity differences between a hardy and less-hardy cultivar of barley was due to specific activity differences rather than the quantity of RNase I present in the samples.

Less than 1/500 of the extracted protein isolated from 3 g of

shoot tissue was used for a rocket immunoelectrophoresis sample. Since approximately 50 4-day-old shoots were used to derive enzyme for these tests, calculations show that each shoot contains more than 10 times the quantity of RNase needed for this method. The adaptation of rocket immunoelectrophoresis to quantitate RNase from a single seedling provides an opportunity for studying the inheritance of RNase activity in barley tissue.

LITERATURE CITED

1. Anraku, Y. 1966. Cyclic phosphodiesterase of Escherichia coli. In: G. L. Cantoni and D. R. Davis. ed., Procedures in Nucleic Acid Research. Harper and Row, New York. pp. 130-143.

2. Clarke, H. G. and T. Freeman. 1966. A quantitative immuno-electrophoresis method (Laurell electrophoresis). Protides of the Biological Fluids. Elsevier, Amsterdam. 14:503-509.

3. Crowle, A. 1973. Immunodiffusion, 2nd ed. Academic Press, New York.

4. Kenefick, D. G., C. Johnson and E. I. Whitehead. 1974. Characteristics of protein synthesis and RNase activity in Hordeum in relation to temperature adaptation. In: R. L. Bielski, A. R. Ferguson and M. M. Cresswell. (eds.), Mechanisms of Regulation of Plant Growth, Bulletin 12. The Royal Society of New Zealand. Wellington, N.Z. pp. 505-511.

5. Kwapinski, V. 1972. Methodology of Immunochemical and Immunological Research. Wiley Interscience, New York.

6. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in antibody-containing agarose gel. Protides of the Biological Fluids. Elsevier, Amsterdam. 14:499-502.

7. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin reagent. J. Biol. Chem. 192: 265-275.

8. Pitt, D. 1971. Purification of a ribonuclease from potato tubers and its use as an antigen in the immunochemical assay of this

protein following tuber damage. Planta (Berl.) 101:333-351.

9. Schaffner, W. and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56:502-514.

10. Tuve, T. W. and C. B. Anfinsen. 1960. Preparation and properties of spinach ribonuclease. J. Biol. Chem. 235:3437-3441.

11. Verbruggen, R. 1975. Quantitative immunoelectrophoretic methods: A literature survey. Clin. Chem. 21:5-43.

12. Wilson, C. M. 1968. Plant Nucleases. I. Separation and purification of the two ribonucleases and one nuclease from corn. Plant Physiol. 43:1332-1338.

13. Wilson, C. M. 1968. Plant nucleases. II. Properties of corn ribonuclease I and II and corn nuclease I. Plant Physiol. 43:1339-1346.

14. Wilson, C. M. 1971. Plant nucleases. III. Polyacrylamide gel electrophoresis of corn ribonuclease isoenzymes. Plant Physiol. 48:64-68.

15. Wilson, C. M. 1975. Plant Nucleases. Annu. Rev. Plant Physiol. 26:187-208.

16. Wong, R. S. 1975. A comparative study of ribonuclease from two winter barley cultivars based upon separation, purification and partial characterization of RNA-degrading enzymes. Ph. D. thesis, South Dakota State University.

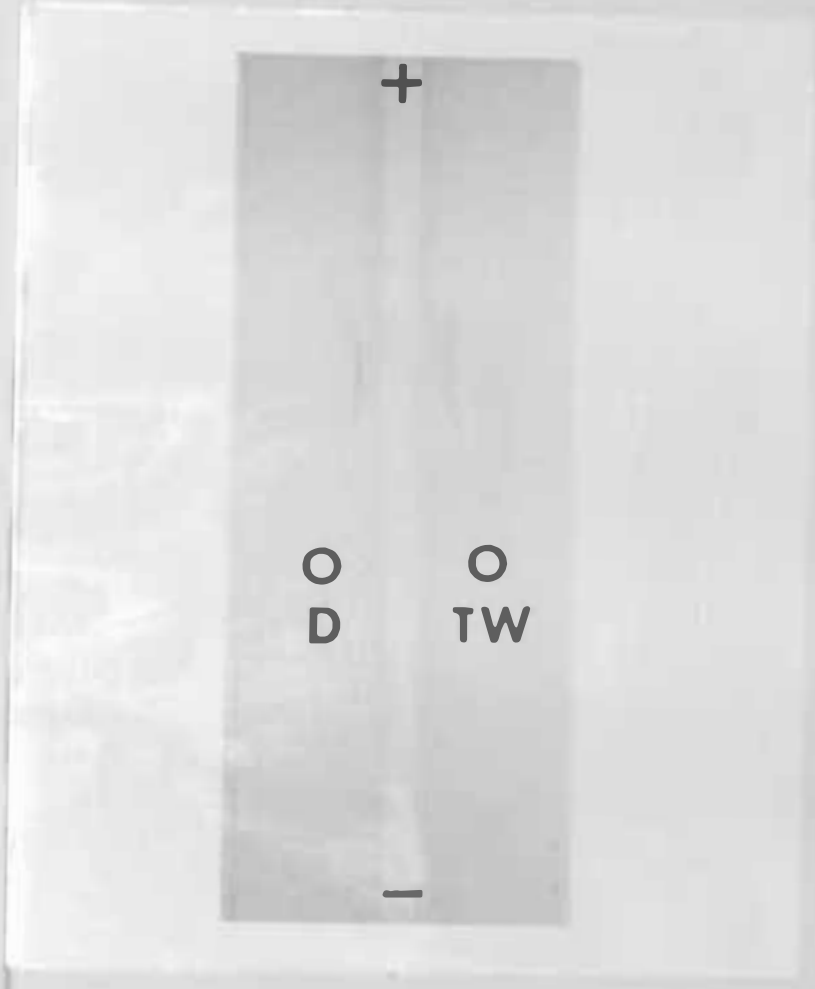

Figure 1. Immunoelectrophoresis of the anionic protein fraction (pH 7.4) from the barley cultivars Tennessee Winter (TW) and Dicktoo (D). The circles indicate the antigen sample wells in the agarose gel. After electrophoresis the antisera trough was formed by removal of the gel and filled with antisera against purified Dicktoo RNase. Similar to results obtained when using purified RNase antigen, a single precipitin arc formed for each protein sample (approx. 0.2 mg crude protein). The results were identical for antibody produced from purified RNase of either cultivar.

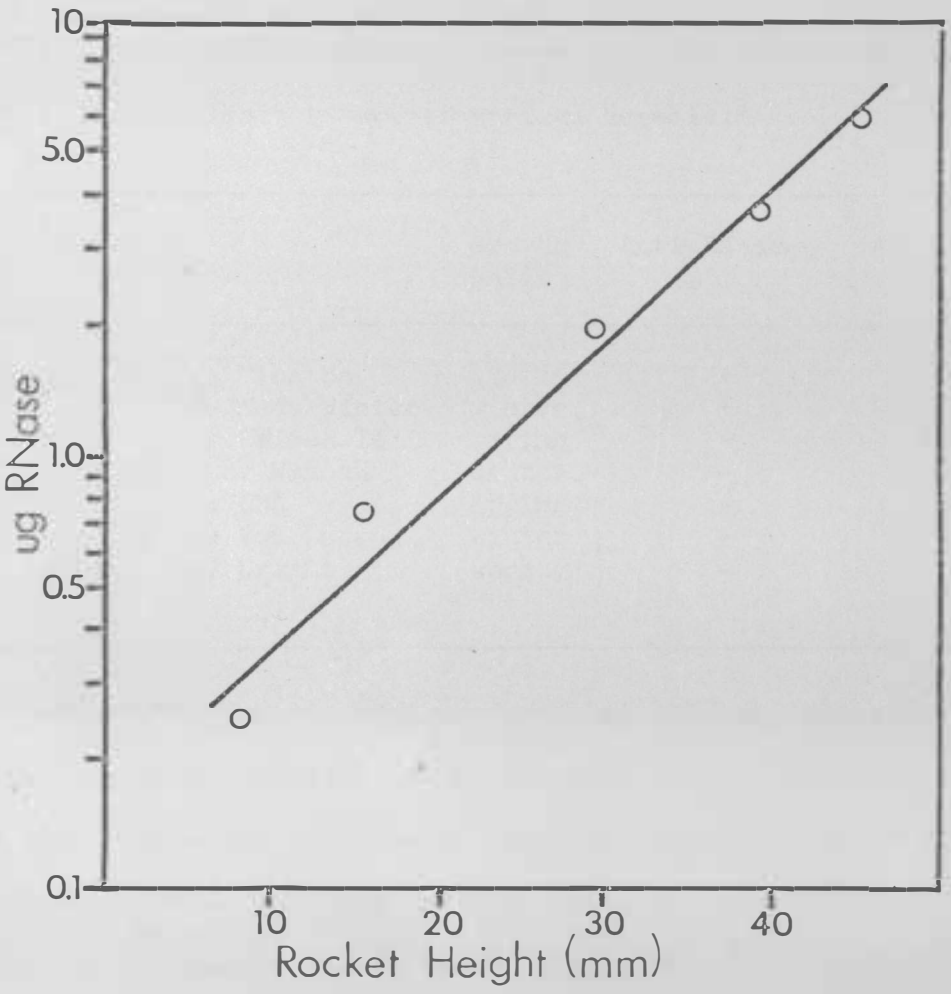

Figure 2. Rocket immunoelectrophoresis standard curve for the purified Tennessee Winter RNase antigen-antibody system. The line drawn represents a least squares fit of the data (correlation coefficient = $r^2 = 0.977$).

Table 1. Specificity of rabbit antisera obtained from the injection of purified RNase from barley cultivars Tennessee Winter (anti-$RNase_{TW}$) and Dicktoo (anti-$RNase_D$) when reacted against soluble protein samples from 4-day-old shoot tissue of various cereals.[1]

Sample source		Growth Habit	Anti-$RNase_{TW}$	Anti-$RNase_D$
Hordeum vulgare	cv Dicktoo	winter	+	+
" "	cv Tenn Winter	winter	+	+
" "	cv Primus II	spring	+	+
Triticum aestivum	cv Winoka	winter	-	-
" "	cv ERA	spring	-	-
Secale cereale	cv Von Lockow	winter	-	-
Avena sativa	cv Wright	spring	-	-

[1]The soluble protein sample of each cereal was prepared by extracting 3 g of 4-day-old shoot tissue in a final volume of 9 ml. Five ml of this sample was desalted on Sephadex G-25 and 6 ml of the eluant showing the highest A_{280} was pooled. Fifty ul (approx. 22 ug crude protein) of the pooled eluant was tested using rocket immunoelectrophoresis. Both antisera were diluted 1:10 in the final gel casting. Reactivity comparable to Tennessee Winter and Dicktoo antigen is indicated by (+). Samples showing no cross-reactivity are indicated with a (-).

Table 2. Specificity of rabbit antisera obtained from the injection of purified RNase from barley cultivars Tennessee Winter (anti-$RNase_{TW}$) and Dicktoo (anti-$RNase_D$) when reacted against soluble protein from 4-day shoot tissue of various Hordeum species.[1]

Sample source	Anti-$RNase_{TW}$	Anti-$RNase_D$
Hordeum vulgare cv Dicktoo	++	++
" " cv Tenn Winter	++	++
" irregulare	++	++
" agrocrithon	++	++
" stebbinsii	+	+
" spontaneum	+	+
" leporinum	+	+
" glaucum	-	-
" marinum	-	-

[1]Fifty ul of a soluble protein sample (Sephadex G-25) extracted from 3 g of 4-day-old shoot tissue of each species was used for the rocket immunoelectrophoresis sample (see Table I). Reactivity similar to the H. vulgare samples is indicated by (++) whereas no reactivity is indicated by (-). Samples indicated by (+) were cross-reactive, but showed signifigantly lower rocket heights than the H. vulgare samples.

Table 3. A comparison of RNase quantity in 4-day-old barley tissue as determined by rocket immunoelectrophoresis.[1]

Cultivar	ug protein/g tissue	Rocket height (mm) Set 1	Rocket height (mm) Set 2	ug RNase/g tissue Set 1	ug RNase/g tissue Set 2
Dicktoo	1530	17.5	17.6	49	49
	1550	18.2	17.8	52	50
Tenn. Winter	1550	18.0	17.5	51	49
	1670	18.0	18.2	51	52

[1]RNase was quantitated using antisera against purified Tennessee Winter RNase and the standard curve in Figure 2. Quantitation of RNase using antisera against purified Dicktoo RNase also showed nearly equivalent quantities in both cultivars. Fifty ul of a soluble protein sample (Sephadex G-25) extracted from 3 g of 4-day-old shoot tissue of each species was used for the rocket immunoelectrophoresis sample (see Table 1). The two sets of data presented represent two RNase determinations using duplicate protein samples from each cultivar.